SUPPLEMENT MATERIAL

Detailed Methods

Generation of *Xirp2* loxP-targeted mice. *Xirp2* loxP-neo targeted mice were generated by inGenious Targeting Laboratory Inc. (Stony Brook, NY) using BAC-mediated recombination placing loxP sites flanking exons 4 and 6 of the *Xirp2* gene. A loxP-flanked PGK-neomycin resistance cassette was inserted between exons 6 and 7. Two targeted embryonic stem cell clones were used to generate chimeric mice, both resulting in germ-line transmission of the targeted allele. EIIa-Cre transgenic mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). All animal procedures were approved by the Institutional Animal Care and Use Committee at Boston University.

Administration of Angiotensin II & Sham Operation. Angiotensin II infusion (0.9µg/hr) was administered via subcutaneous osmotic mini-pumps (Alzet model 2004) for 14 days to wt (n=8) and *Xirp2* hypomorphic (n=6) mice. Pumps were implanted under anesthesia with ketamine/xylazine (100mg/kg/10mg/kg). Sham-operated animals (n=4 wt, n=3 hypomorph) were implanted with empty mini-pumps under identical conditions.

Echocardiography and blood pressure analysis. Transthoracic M-mode echocardiography was performed on mice anesthetized with 1-2% isofluorane at baseline and post-2week AngII infusion. The heart rate was maintained between 500-600 beats per minute. Blood pressure analysis was performed using the non-invasive tail cuff method (Model BP 2000, Visitech Systems). Systolic blood pressure and heart rates were measured daily. Post-2week readings were determined using measurements recorded on day 14 post-AngII treatment or shamoperation.

Histology. Hearts were fixed in 4% paraformaldehyde for 1 hour at 25°C, cryoprotected in 30% sucrose at 4°C, placed in embedding compound (OCT), snap-frozen and stored at -80°C. Whole-heart sections were stained with hematoxylin & eosin (H&E) for visualization of general cardiac structures. Masson's trichrome staining was performed to determine the extent of cardiac fibrosis. Fibrosis quantification was performed using a Matlab software (Mathworks, Natick, MA) image processing algorithm which determined % fibrotic (blue) pixels out of total tissue pixels per image. Apoptosis was detected by terminal dUTP nick-end labeling (TUNEL) assay using the Promega DeadEnd™ Colorimetric TUNEL System kit. Heart sections for TUNEL assay were prepared according to manufacturer's instructions. Extent of apoptosis (apoptotic index) was quantified using a separate Matlab software algorithm to quantify the % of apoptotic nuclei pixels out of total tissue pixels per image. Cross-sectional area (CSA) of ventricular muscle was assessed by Metamorph software (Molecular Devices, Downington, PA) of vinculin-stained cardiac sections (see *Immunofluorescence*) and by wheat germ agglutinin (WGA) staining (see *Wheat germ agglutinin staining)*. Heart weight: body weight ratios (HW: BW) were measured as follows: after animal sacrifice, whole-body weight was measured and recorded in kilograms (kg) and hearts weights were measured and recorded in grams (g).

Immunofluorescence. 10µm heart cryosections were blocked for 1 hour (3%BSA in 1XPBS) at 25°C, incubated for 1 hour with mouse monoclonal anti-vinculin antibodies (Sigma) (1:200), incubated for 1 hour in secondary anti-mouse Alexa-Fluor (Invitrogen) antibodies (1:500), and mounted with Vectashield medium (Vector Labs).

Wheat germ agglutinin staining. Cardiomyocyte cross-sectional area (CSA) was analyzed by staining cardiac cryosections with wheat germ agglutinin (WGA)-Alexa Fluor 488 conjugate (Invitrogen). Cryosections were first washed in 1XPBS and then incubated in 10μg WGA-Alexa Fluor 488 for 1 hour at room temperature followed by additional washes in 1XPBS. Slides were mounted with Vectashield mounting medium (Vector Labs) and sealed. CSA was determined by analyzing WGA-stained images with Metamorph software (Molecular Devices, Downington, PA).

RT-PCR/qRT-PCR. Either hindlimb or ventricular RNA was extracted using Trizol reagent (Invitrogen) and cDNA was prepared from total RNA using random hexamers and M-MLV reverse transcriptase (Promega). Primers for qRT-PCR/RT-PCR are: *Xirp2*(exons6/7)-RTPCR: 5'-cccagccaagtgtatgaagattgaa-3'/5' actgcacctctcctttgaggatttc-3'; *Xirp2(*exons7/8)-RTPCR: 5'-gtcatctctggccacatcct-3'/5'-tgatggcatctgaagcaaga-3'; *Xirp2*(exons2/3)-qRT-PCR: 5'- gcagcttctcggctaatgtca 3'/5'-aggcgttgcaggttgaagtc-3'; *Xin*-qRT-PCR: 5'-gctccggcgtctctacaaac- 3'/5' ccagcgcatacactgaacatc-3'; *αMHC*-qRT-PCR: 5'-gcccagtacctccgaaagtc-3'/5'- gccttaacatactcctccttgtc-3'; *βMHC*-qRT-PCR: 5'-actgtcaacactaagagggtca-3'/5'- ttggatgatttgatcttccaggg-3'; *ANF*-qRT-PCR: 5'-acctgctagaccacctggaggag-3'/5'- ccttggctgttatcttcggtaccgg-3'; *BNP*-qRT-PCR: 5'-atctcctgaaggtgctgtcccag-3'/ 5'- ggtcttcctacaacaacttcagtgcgttac-3'; β-2-*Microglobulin(B2M)*-qRT-PCR: 5'- ctgcagagttaagcatgccagt-3'/5'-tcccagtagacggtcttggg-3'; *MARCKS*-qRT-PCR: 5'- ggccgaaaaggatgaggct-3'/5'-agcttgaaggacttcttgaagga-3'; *Lipocalin2*-qRT-PCR: 5'- gattcgtcagctttgccaagt-3'/5'-cattggtcggtgggaacag-'3; *Pdlim3/ALP*-qRTPCR: 5'- tgggggcatagacttcaatca-3'/5'-ctccgtaccaaagccatcaatag-3'; *RCAN1/MCIP1-qRT*-PCR: 5'- ggctgcacaagaccgagtt-3'/5'-gggggtggcatcttctactt-3'; *AT1R-RT-PCR:* 5' caagaaagccatcaccagatca- 3'/5'-gctgtttccaaatattcccacc-3'; *GAPDH*-RT-PCR: 5' gccatcaacgacccttcattg-3'/5'- actccacgacatactcagcacc-3'. For qRT-PCR, individual non pooled samples were run in triplicate wells. qRT-PCR was performed with SYBR® Green master mix (Applied Biosystems) using the 7900 Sequence Detection System (Applied Biosystems).

Microarray. RNA from wt and hypo heart ventricles were prepared as above, quantified individually, and pooled in equimolar concentrations (n=8 AngII-wt, n=6 AngII-hypo, n=3 wt, n=3 hypo). Samples were prepared as have been described previously (1) and hybridized to the Mouse Gene 1.0 ST Array (Affymetrix) at the Boston University Microarray Facility.

Western blot analysis. To detect Xirp2 protein in the heart, ventricular muscle was snapfrozen in liquid nitrogen immediately following dissection. Tissue was pulverized and resuspended in sample loading buffer (5mmol/L EDTA; 30% Glycerol; 60mmol/L Tris-HCl pH 6.8; 15% SDS; 7.5% beta-mercaptoethanol; and 0.1% bromphenol blue). Samples were heated at 55ºC for 30 minutes followed by sonication, then incubated again at 55ºC for 30 minutes. To detect Xirp2 protein in skeletal muscle, hind limb muscle was lysed in ELB buffer [50mmol/L HEPES, pH 7.0; 250mmol/L NaCl, 5mmol/L EDTA, 0.1% IGEPAL, 1mmol/L DTT, 1mmol/L PMSF, and protease inhibitor mixture (Complete, Roche Biochemical)]. Tissue was incubated in ELB buffer for 10 minutes on ice, and centrifuged to yield soluble protein extracts. Protein concentrations were analyzed by Bradford assay. Samples were subjected to SDSPAGE, transferred to PVDF membrane (Biorad) and immunoblotted using rabbit polyclonal anti-Xirp2 antibodies (1:1000) which have been described previously (2). For detection of PS9-GSK-3β in mouse heart, extracts were prepared as above and immunoblotted with either mouse monoclonal anti- total GSK-3β (1:3000) (BD Biosciences), or rabbit polyclonal

anti-PS9-GSK-3β (1:5000) (Cell Signaling). Type I angiotensin II receptor (AT1R), β-catenin, and RCAN1/MCIP1 were detected using rabbit polyclonal anti-AT1R (1:200) (Santa Cruz Biotech), mouse anti-β-catenin (1:1000) (BD Transduction Laboratories), or rabbit polyclonal anti-MCIP1 (1:250) which was described previously (3). Phospho Serine-473-Akt and total Akt were detected with rabbit polyclonal Cell Signaling antibodies (1:1000), and MEF2A was detected using rabbit polyclonal (c-21) antibodies from Santa Cruz Biotech. (1:500). Immunoblots were washed and incubated with HRP-conjugated secondary antibodies (1:2000) and reacted with Western Lightning chemiluminescent reagent (Perkin Elmer).

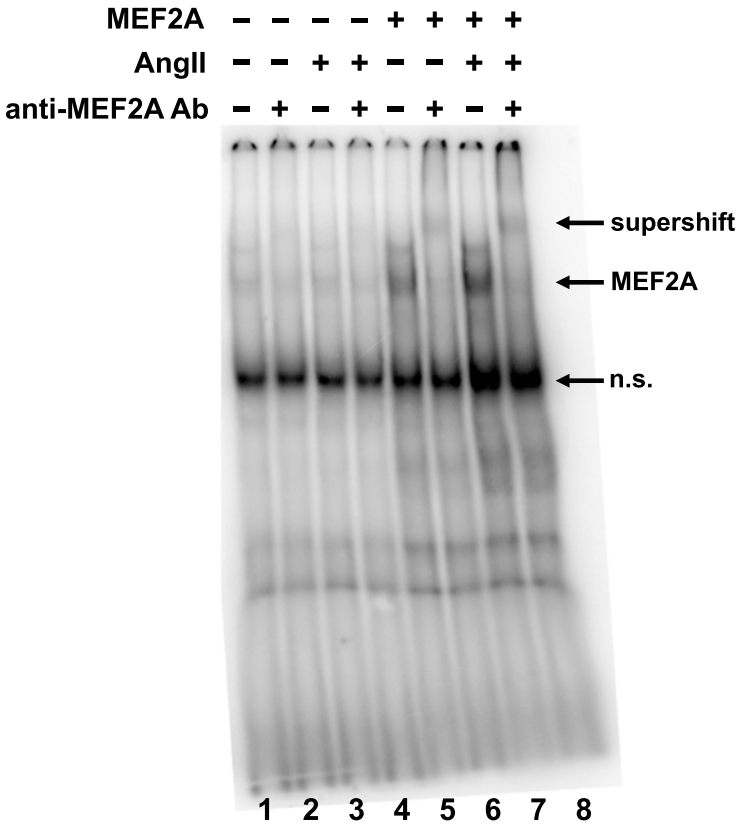
Plasmids. For luciferase assays, the MEF2A expression plasmid used was pcDNA1-MEF2A containing the human cDNA. All *Xirp2* luciferase promoter constructs were cloned into the pGL3b-luciferase vector (Promega) except the -1425/-285 deletion mutant, which was cloned into the pGL3p-luciferase construct (Promega).

Cell culture & luciferase assays. COS1 kidney fibroblast cells were grown in 6-well plates using Dulbecco's modified Eagle medium with 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin, and 1% L-Glutamine. Cells were transfected with 1.5 μg total DNA using Mirus TransIT®-LT1 transfection reagent. 24 hours post-transfection, cells were washed in 1XPBS, and media was switched to either serum-free DMEM or serum-free DMEM with 10μmol/L human AngII (Calbiochem). Luciferase assays were performed 48 hours posttransfection using Luciferase Assay Reagent (Promega), and results were normalized by Bradford assay. For analysis of *Xirp2* expression in primary neonatal rat ventricular myocytes (NRVM), cells were isolated as described previously (2). Cells were seeded in 60 mm dishes at a density of $1x10_6$ cells and maintained for 24 hours in DMEM containing 10% FBS, 2mmol/L L-glutamine, and 1% penicillin-streptomycin. After 24 hours, the media was replaced with DMEM containing one of the following treatments: 0.5X Nutridoma-SP (Roche), or 0.5X Nutridoma-SP plus 10μmol/L AngII. Cardiomyocytes were incubated for 72 hours, and then harvested for RNA extraction as described above.

Generation of shRNAs & knockdown in NRVMs. Short-hairpin RNAs (shRNA) were designed by using the BLOCK-iT™ RNAi designer system (Invitrogen) to generate double-stranded oligonucleotides targeting either *Mef2a* or *LacZ*. These oligonucleotides were cloned into the pENTR™/U6 RNAi entry vector (Invitrogen) for use in transient transfection assays testing knockdown efficacy. shRNAs were then excised from the pENTR™/U6 vector and ligated into the pAd/BLOCK-iT-DEST destination vector for the generation of adenovirus. Recombinant $pAdMEF2A_{shRNA}$ and $pAdLacZ_{shRNA}$ adenoviral vectors were linearized and transfected into sub-confluent 293A cells for viral propagation. Crude adenovirus was harvested from A293 cells 10-13 days following infection, and purified using cesium chloride centrifugation. Purified adenoviruses containing *Mef2A* and *LacZ* shRNAs were then used at an MOI (multiplicity of infection) of 20 to transduce NRVMs over the course of 36 hours.

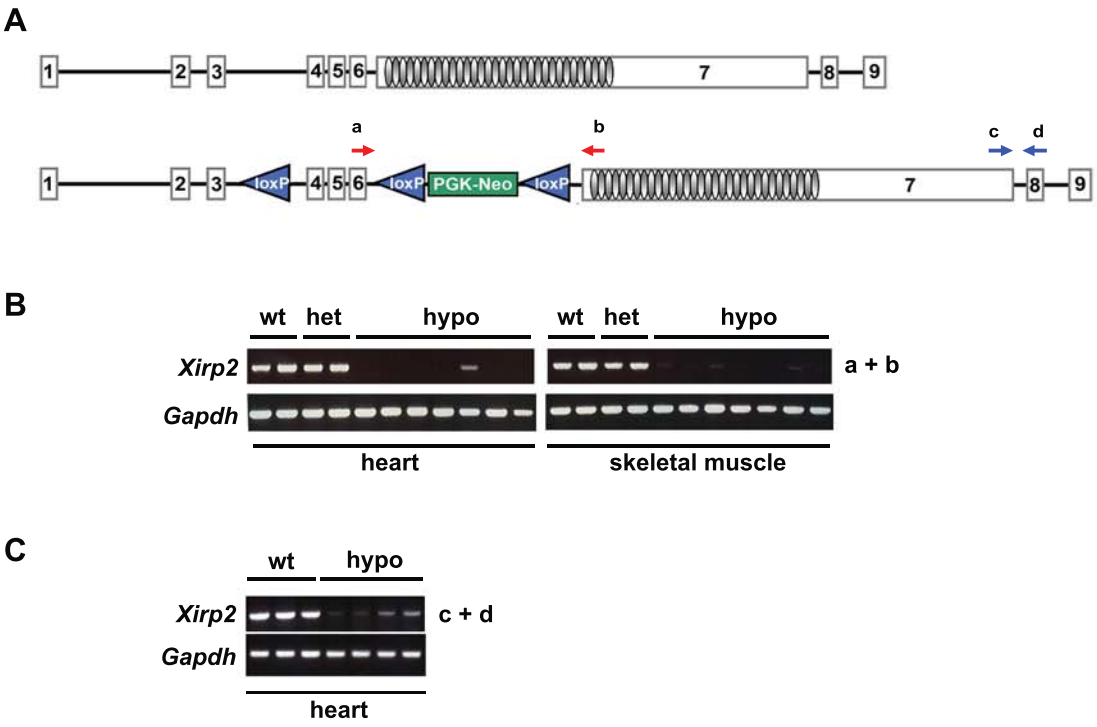
Electrophoretic mobility shift assays. Detection of protein-DNA complexes by gel shift was carried out using protein extracts from MEF2A-transfected COS cells which were cultured in the presence or absence of 10μmol/L AngII. Procedures for gel shift have been described previously (1). Supershift of MEF2A-DNA complexes was performed by pre-incubation of protein extracts with anti-MEF2A antibodies (c-21) (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Online Figure 1



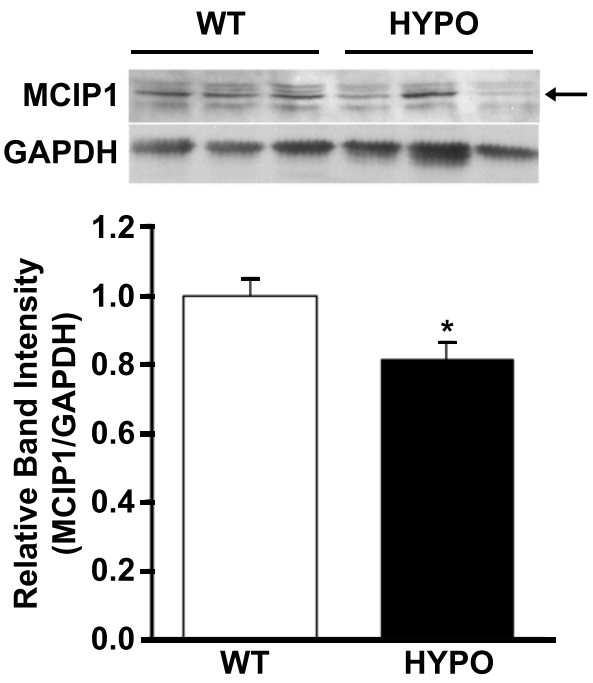
Online Figure 1. AngII-stimulation of MEF2A on the *Xirp2* promoter is not mediated by enhanced DNA binding. MEF2A from transfected COS cells shifts the mobility of the -75 MEF2 site oligo (lane 5). Confirmation of MEF2 binding is determined (lane 6) by addition of MEF2A antibodies. Addition of 10 μmol/L AngII to COS cells does not result in enhanced DNA binding (lanes 7, 8). Presence of non-specific band (n.s.) indicated by arrow below MEF2A binding. Modestly enhanced MEF2A-protein/DNA complex in lane 7 is attributable to overall enhanced signal in lane 7 (see enhanced intensity of non-specific band n.s.). Results representative of multiple experiments.

Online Figure 2



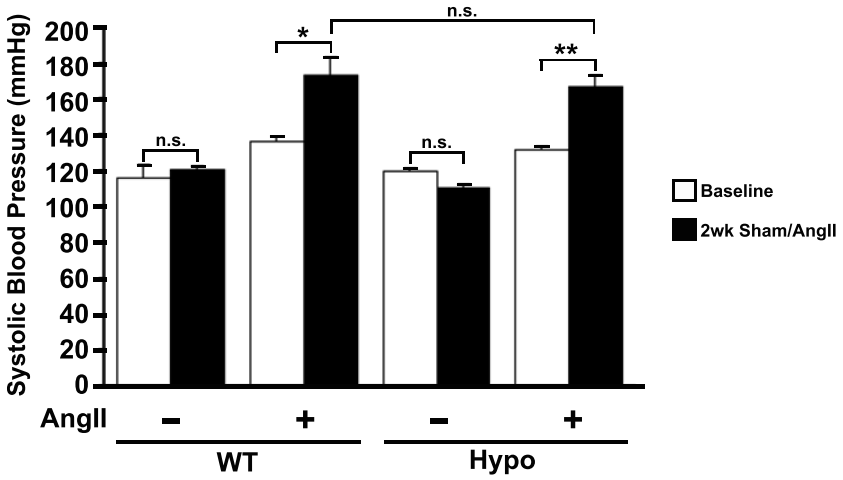
Online Figure 2. Hypomorphic mice display dramatically reduced expression of normal sized transcripts as assayed by multiple RT-PCR primer sets. (A) Wild type (wt) *Xirp2* allele shown (top), targeted hypomorphic (hypo) loxP-neo locus (bottom) with RT-PCR primers for spanning exons 6/7 (a+b) and exons 7/8 (c+d). **(B)** RT-PCR result for *Xirp2* expression using primers (a+b) with heart cDNA (left panel) and hindlimb cDNA (right panel). *Gapdh* internal control. n=2 wt, n=2 het, n=7 hypo. **(C)** RT-PCR result for *Xirp2* expression using primers (c+d) with heart cDNA. *Gapdh* internal control. n=3 wt, n=3 hypo.

Online Figure 3



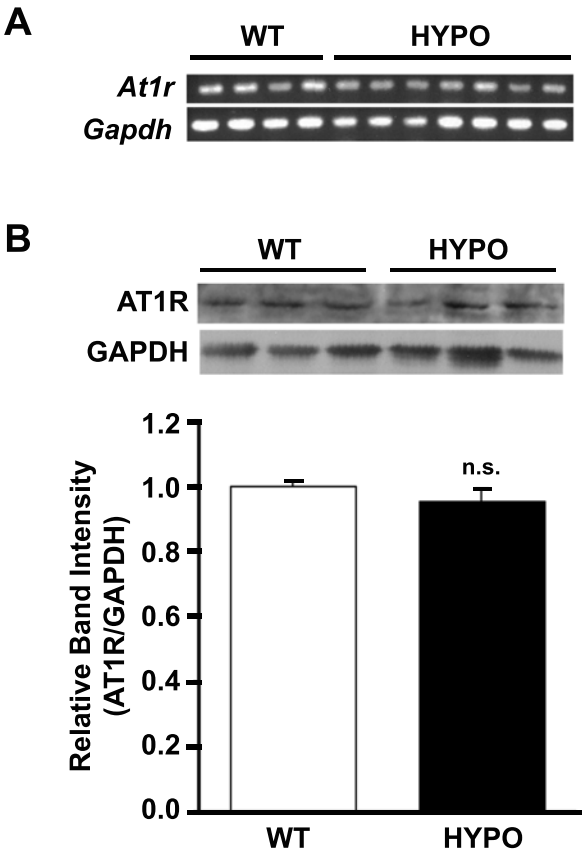
Online Figure 3. Hypomorphic mice show reduced cardiac protein expression of MCIP1. Western blot (upper panel) for MCIP1 (arrow indicates position of MCIP1 band) is reduced in hypo hearts, n=3 wt, n=3 hypo. GAPDH loading control. Quantification of MCIP1 protein expression (lower panel) normalized to GAPDH expression using ImageJ software. Mean MCIP1 expression graphed relative to wt. n=3 wt, n=3 hypo. Cardiac hypo MCIP1 expression reduced by 20% (*$p<0.05$). Error bars indicate ±SEM.

Online Figure 4



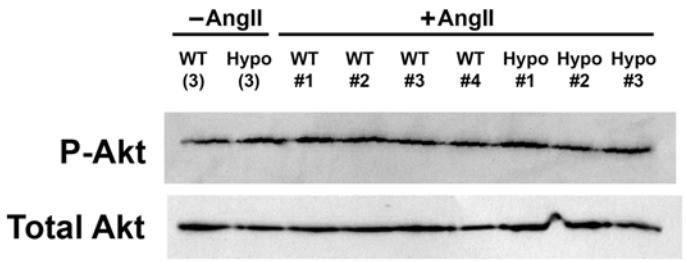
Online Figure 4. Hypertension following 2-week AngII treatment in wild type and *Xirp2* hypomorphic mice. Systolic blood pressure (bp) measurements, baseline (white bars), post-2week AngII-treatment or sham operation (black bars). 2week AngII-treatment of wt mice vs. baseline wt bp (173mmHg vs. 136mmHg, *p<0.05), 2week AngII-treatment of hypo mice vs. baseline hypo bp (167mmHg vs. 132mmHg, **p<0.005), 2week AngII-wt vs. 2week AngII-hypo (n.s., p>0.05), post-sham-wt vs. baseline sham-wt (n.s., p>0.05), post-sham-hypo vs. baseline sham-hypo (n.s., p>0.05). n=4 sham wt, n=3 sham hypo, n=8 AngII-wt, n=6 AngII=hypo. Error bars indicate ±1SEM.

Online Figure 5



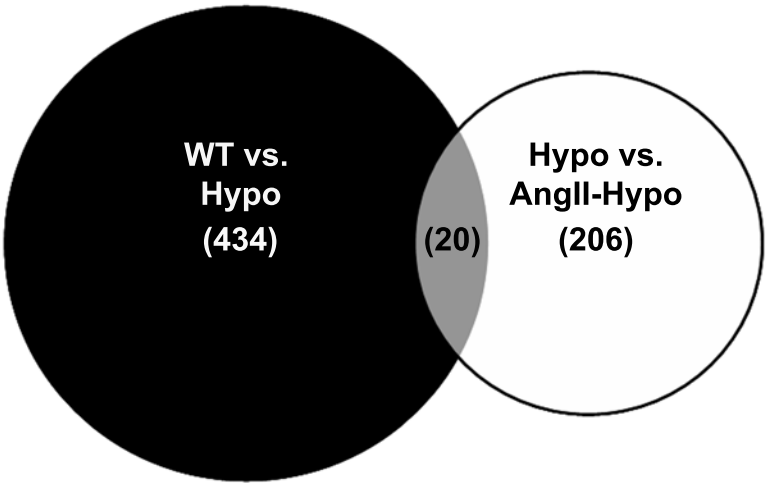
Online Figure 5. *Xirp2* hypomorphic mice express normal levels of the Angiotensin type I receptor (AT1R). **(A)** RT-PCR analysis of *At1r* expression in wt and hypo hearts, n=3 wt, n=3 hypo. *Gapdh* internal control. **(B)** Western blot (top panel) for AT1R protein in wt and hypo hearts, n=3 wt, n=3 hypo. GAPDH loading control. Quantification of Western blot (bottom panel) for AT1R normalized to GAPDH expression for each sample, bands quantified using ImageJ software. Average AT1R protein levels not were significantly different (n.s.) between wt and hypo mice. Error bars indicate ±1SEM.

Online Figure 6



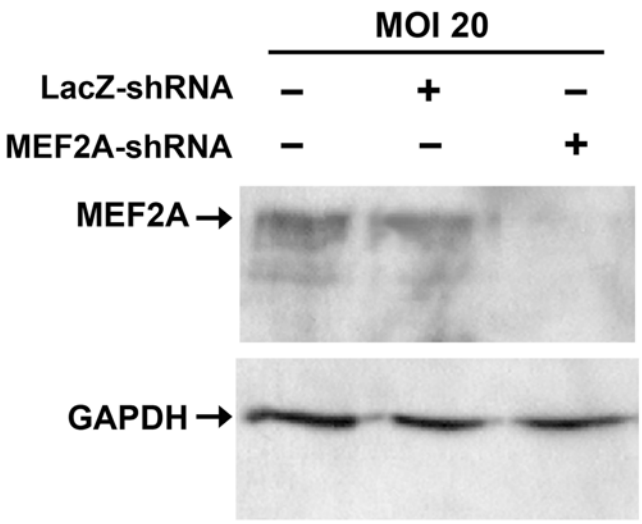
Online Figure 6. Levels of phosphorylated Akt are not altered in hypomorphic mice treated with Angiotensin II. Western blot analysis of levels of P-Akt (phosphorylated on serine-473) and total Akt using cardiac protein extracts from untreated wt (pooled, n=3), untreated hypo (pooled n=3), and individual AngII-wt (n=4) and AngII-hypo (n=3) mice.

Online Figure 7



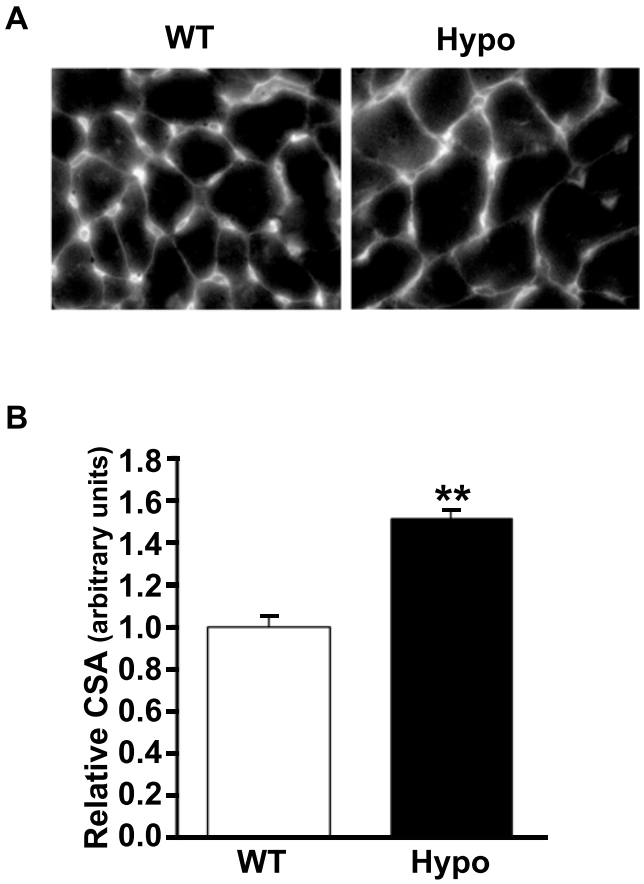
Online Figure 7. Uniquely dysregulated cardiac gene profiles highlight the distinct functions of Xirp2 in unstressed and AngII-stressed conditions. A comparison of genes 2-fold or more dysregulated from the microarray between those dysregulated in the unstressed hypo (wt vs. hypo) and the AngII-treated hypo (hypo vs. AngII-hypo). 434 unique transcripts were dysregulated in hypo vs. 206 unique transcripts dysregulated in AngII-hypo, 20 of which were dysregulated in both comparisons.

Online Figure 8



Online Figure 8. Adenovirus-delivered-MEF2A-shRNA specifically and effectively knocks down MEF2A protein in NRVMs. Western blot for either MEF2A (top panel) or GAPDH (lower panel) using NRVM protein extracts which were either untransduced, transduced with adenovirus delivering the LacZ-shRNA, or transduced with adenovirus deliving the MEF2A-shRNA. MEF2A protein expression greatly reduced as a result of MEF2A--shRNA-mediated knockdown but not with LacZ-shRNA. GAPDH serves as internal control. Multiplicity of infection (MOI) of 20 used for both LacZ-shRNA and MEF2A-shRNA.

Online Figure 9



Online Figure 9. Cross-sectional area analysis using wheat germ agglutinin staining reveals cardiac hypertrophy in hypomorphic hearts. (A) Cross-sections of wt and hypo left ventricle stained with wheat germ agluttinin (WGA)-alexa-fluor-488 conjugate. n=3 wt, n=3 hypo. **(B)** Cross-sectional area (CSA) analysis of WGA-stained images using Metamorph software reveals 1.5-fold increase in hypo vs. wt (**p<0.005). Error bars indicate +/-1SEM.

Online Table I

		WT (n=8)		HYPO (n=6)	
Measurement/ Calculation	**Units**	**Avg**	**Stdev**	**Avg**	**Stdev**
%EF	%	69.87	18.65	70.51	18.26
% FS	%	41.17	15.35	41.05	13.62
IVS;d	mm	0.86	0.19	0.81	0.13
IVS;s	mm	1.44	0.30	1.38	0.28
LVPW;d	mm	0.90	0.11	0.88	0.14
LVPW;s	mm	1.37	0.22	1.43	0.34
LVID;d	mm	3.73	0.34	3.64	0.35
LVID;s	mm	2.21	0.70	2.18	0.70
LV Vol;d	µl	59.80	12.39	56.50	12.59
LV Vol;s	µl	19.05	14.47	18.28	15.07
LV Mass	mg	119.23	19.08	108.61	15.50
LV Mass Corrected	mg	95.38	15.26	86.88	12.40

Online Table I. Hypomorphic mice do not show altered cardiac function as measured by echocardiography. Echocardiographic parameters show no significant difference between unstressed wild type and *Xirp2* hypomorphic mice. The average value for each parameter for wt (n=8) and hypo (n=6) mice is displayed along with 1 standard deviation (Stdev). EF, ejection fraction. FS, fractional shortening. IVS;d, interventricular septum;diastole. IVS;s, interventricular septum;systole. LVPW;d, left ventricle posterior wall;diastole. LVPW;s, left ventricle posterior wall;systole. LVID;d, left ventricular internal dimension;diastole. LVID;s, left ventricular internal dimension;systole. LV Vol;d, left ventricle volume;diastole. LV Vol;s, left ventricle volume;systole. LV Mass, left ventricle mass.

Supplemental References

1. Durham JT, Brand OM, Arnold M, Reynolds JG, Muthukumar L, Weiler H, Richardson JA, Naya FJ. Myospryn is a direct transcriptional target for MEF2A that encodes a striated muscle, alpha-actinin-interacting, costamere-localized protein. *J Biol Chem*. 2006;281:6841-9.

2. Huang HT, Brand OM, Mathew M, Ignatiou C, Ewen EP, McCalmon SA, Naya FJ. Myomaxin is a novel transcriptional target of MEF2A that encodes a Xin related alpha-actinin-interacting protein. *J Biol Chem*. 2006; 281:39370-9.

3. Sanna B, Brandt EB, Kaiser RA, Pfluger P, Witt SA, Kimball TR, van Rooij E, De Windt LJ, Rothenberg ME, Tschop MH, Benoit SC, Molkentin JD. Modulatory calcineurin-interacting proteins 1 and 2 function as calcineurin facilitators in vivo. *Proc Natl Acad Sci U S A*. 2006;103:7327-32.